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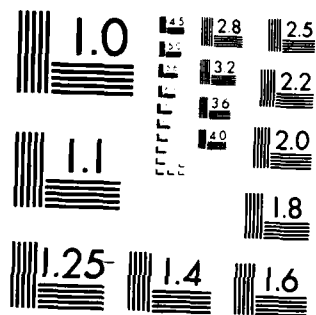   ANATOMY AND BIOLOGY PHILADELPHIA PA M A BRINTON JUN 80
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ANALYSIS OF FLAVIVIRUS REPLICATION

Annual Summary Report

Margo A. Brinton, Ph.D.

June, 1980

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Even though the flavivirus group represents one of the largest groups of viruses known to cause significant human disease, little is yet understood about the replication cycle of flaviviruses. Comparative studies are carried out in genetically resistant C3H/RV and genetically susceptible C3H/HE murine embryofibroblast cultures. These studies have indicated that host factor(s) can modulate the level of flavivirus RNA synthesis.

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Report:

The overall objective of this research is to characterize the structure of the flavivirus genome RNA and to further describe the mode of its transcription and translation. The best characterized togaviruses both belong to the alphavirus group and very little is currently known about the mode of flavivirus replication. Recent studies (1-3) have indicated that flaviviruses differ significantly from alphaviruses in their mode of replication. Specifically, no subgenomic m-RNA molecule such as the alphavirus 26S RNA has been identified in flavivirus-infected cells and preliminary evidence indicates the possibility of internal initiation and termination events during translation of flavivirus proteins. Therefore, it seems imperative to the understanding and eventual control of flavivirus-induced disease to study the replication of flaviviruses directly and not to rely on assumptions drawn from alphavirus data.

Even though many flaviviruses are the cause of a significant amount of morbidity and mortality in the Mediterranean area, Asia, South and Central America and Africa, the selection of safe, effective vaccines for flaviviruses has proved difficult. The only flavivirus human vaccine currently available is for yellow fever. A more thorough understanding of the flavivirus replication cycle will provide clues which should prove useful to the preparation of future vaccines. Also, information about the types of intermediate molecules involved in flavivirus replication and the development of techniques to analyze these intracellular virus-specific molecules will speed analysis of future candidates for vaccine strains. Information about the mechanism by which genetically resistant mouse cells can interfere with the replication of flaviviruses could lead to future effective chemotherapy for flaviviruses.

Although more than 50 different flaviviruses have been identified by serological methods, most replicate poorly in tissue culture systems and, to date, relatively little is known about their replication cycle or the specifics of their interaction with the host cell.

West Nile virus has been chosen as a model flavivirus for this research project, because it is able to grow to sufficient titers in many types of tissue culture cells to make feasible the proposed experimental procedures. In addition, West Nile virus has been chosen because of the similarity of the disease it induces to that caused by Dengue viruses and also because the investigator has had extensive experience in the propagation of West Nile virus. In the future, the techniques used to study West Nile virus will be further modified to make them applicable for confirming studies with such viruses as Dengue and Yellow Fever which are more significant human pathogens but which replicate to lower titers in tissue culture systems.

[1] Wengler, G., Wengler, G., and Gross, H.J. 1978. Virology 89:423-437.
[2] Westaway, E.G. 1977. Virology 80:320-335.
[3] Wengler, G., Beato, M., and Wengler, G. 1979. Virology 96:516-529.

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During the contract period, October 1, 1979 to January 15, 1980, studies were carried out to determine the optimal conditions for labeling flavivirus-specific RNA and protein with radioactive precursors. Initial experiments were done in BHK cells and subsequent ones in mouse embryo fibroblast cultures prepared from congenic C3H mouse strains which are genetically resistant (RV) or susceptible (HE) to flaviviruses. The E101 strain of West Nile virus (WNV) and the 17-D strain of Yellow Fever virus (YFV) were used for these experiments.

The WNV genome (42S) was detected in infected cells after labeling with ^{3}H-uridine in the presence of Act D as early as 4 hr after infection. Various amounts of low molecular weight RNAs (8-10S) were also observed to be synthesized during the first 12 hours. These species were not observed in cell extracts from uninfected control cultures, so they are either virus-specific or cellular mRNAs whose production is stimulated by virus infection. Species of RNA which turned over rapidly were observed in infected cell extracts sedimenting between 22-26S. It is likely that this peak of RNA represents replicative intermediate (RI) structures. Identification of these RNA species will be attempted. Initially, the sensitivity of this peak to RNase will be tested. LiCl precipitation, denaturation and hybridization techniques will be used for further analysis to determine the degree of double-standardness of these molecules.

The analysis of WN virion RNA harvested from resistant and susceptible cells at various times after infection have yielded some very interesting data. At 24, 28 and 72 hr after infection, culture fluid was removed from WNV-infected RV, HE and BHK cell cultures and fresh media containing ^{3}H-uridine was added. Six hours later, the fluid was harvested, clarified and virus was pelleted at 80,000 x g for 2 hr. The pelleted virus was banded on sucrose gradients, treated with detergent and pronase, extracted with phenol three times and precipitated with ethanol. RNA was subsequently analyzed under denaturing or nondenaturing conditions on 2% agarose slab gels. The RNA of extracellular WNV grown in HE or BHK was predominantly 42S genome RNA and increased in amount with time after infection. The amount of 42S RNA in extracellular virions also increased in RV culture fluids with time, even though infectivity titers were lower at later times after infection as compared to comparable HE cultures.

As can be seen in Figure 1 (panel A), a second peak of a faster migrating RNA was visible in WN virions harvested from resistant cells 48 hr after infection. The synthesis of this RNA was increased during the 72-hr labeling period and at this time, a third peak of RNA was also visible (panel B). The WN virions from susceptible cell cultures did not appear to contain these unique species in detectable amounts, although a slight shoulder was observed on the 42S RNA peak obtained at 72 hr from WN virions grown in susceptible cells (panel C). It is not presently known whether or not the faster migrating RNAs isolated from WN virions grown in resistant cells are specifically deleted viral genomes. If so, these RNAs may represent the genomes of defective interfering particles. Experiments are in progress to test this hypothesis. C-DNA to WNV genome RNA as well as specifically end-labeled RNA probes are being prepared to assess the sequence homology of the observed subgenomic RNAs.

Considerable effort was expended to find suitable conditions for labeling and analyzing intracellular proteins in extracts of resistant and susceptible cells at various times after infection with WNV or YF. A number of treatments, such as salt shock or the use of various combinations of inhibitors, were tried in attempts to visualize WNV- and YF-specific proteins in cell extracts. Variation in protein

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bands between experiments has made interpretation of these analyses difficult. However, we have been able to identify virus-specific polypeptides on 5-20% SDS-acrylamide gradient gels, and on 10% SDS-acrylamide gels.

In recent experiments, a unique large molecular weight protein (90,000 daltons) was observed in RV cell extracts labeled for 15 min at 72 hr after infection. This protein band was not observed in susceptible cell or uninfected cell extracts.

The expected viral-specific proteins, structural and non-structural, were observed to be in higher concentrations in HE cell extracts at all time periods as compared to RV extracts. This probably indicates that viral proteins are synthesized more efficiently in susceptible cells. Proteins were labeled with ^{35}S-methionine for 15 min at various times after infection with WNV. Cell cultures were incubated in methionine minus medium with actinomycin D (2 μg/ml) for 3 hr prior to addition of the radioactive precursor. DNA present in the extracts was sheared by sonication and samples were electrophoresed on 10% acrylamide slab gels with 5% stacking gels using the Laemmili (4) buffer system.

We hypothesize that the unique protein detected by 72 hr after infection in RV cell extracts, might represent a gene product of a specifically deleted WNV genome. The deletion may cause an aberrant cleavage of the translation products of this RNA. However, it is currently not known whether flavivirus translation initiates at only a single site at the 5' end of the molecule or at multiple internal sites. Inhibitor studies have so far not allowed the visualization of any polyprotein precursor molecules. An alternative possibility is that a normal viral precursor molecule accumulates in resistant cells due to blockage of the viral protein cleavage mechanism in these cells. The gene product of the resistance gene may act at this level. Experiments will be carried out to characterize this protein.

Virion structural proteins have been separated on preparative SDS-acrylamide slab gels, extracted and used to elicit antiserum. These sera will be employed in future experiments to precipitate viral structural proteins from extracts of resistant and susceptible cells harvested at various times after flavivirus infection. Experiments are also in progress to prepare an antiserum which will bring down non-structural virus-specific proteins from infected cell extracts. Hybridomas producing monoclonal antibodies to specific determinants of flavivirus proteins will also be selected.

Serial high moi passage experiments, interference experiments, and, now, preliminary biochemical experiments (see above) all indicate the production of flavivirus DI particles. However, to date, attempts to physically separate DI particles from standard virions on gradients have been unsuccessful. Renografin gradients are currently being tried.

Several experiments were conducted to determine whether or not WNV could establish a persistent infection in resistant and/or susceptible cells. Such cultures are of interest, because it has been found with several other virus

[4] Laemmili, U.K. 1970. Nature 227:680-685.

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systems that persistently infected cultures produce DI particles and, in some cases, also temperature sensitive virus mutants. Several lines of persistently infected cultures of RV and HE cells have been prepared. The production of infectious virus appears to be cyclic with passage. Virus from cells persistently infected for 15 to 20 passages is currently being analyzed. Intracellular virus-specific RNA synthesis is also being investigated in these cells.

The data so far obtained suggests that interesting insights about the mode of flavivirus replication, as well as about the types of interactions which occur between flaviviruses and their host cells will result from further analysis of what appear to be specifically deleted flavivirus DI genomes.
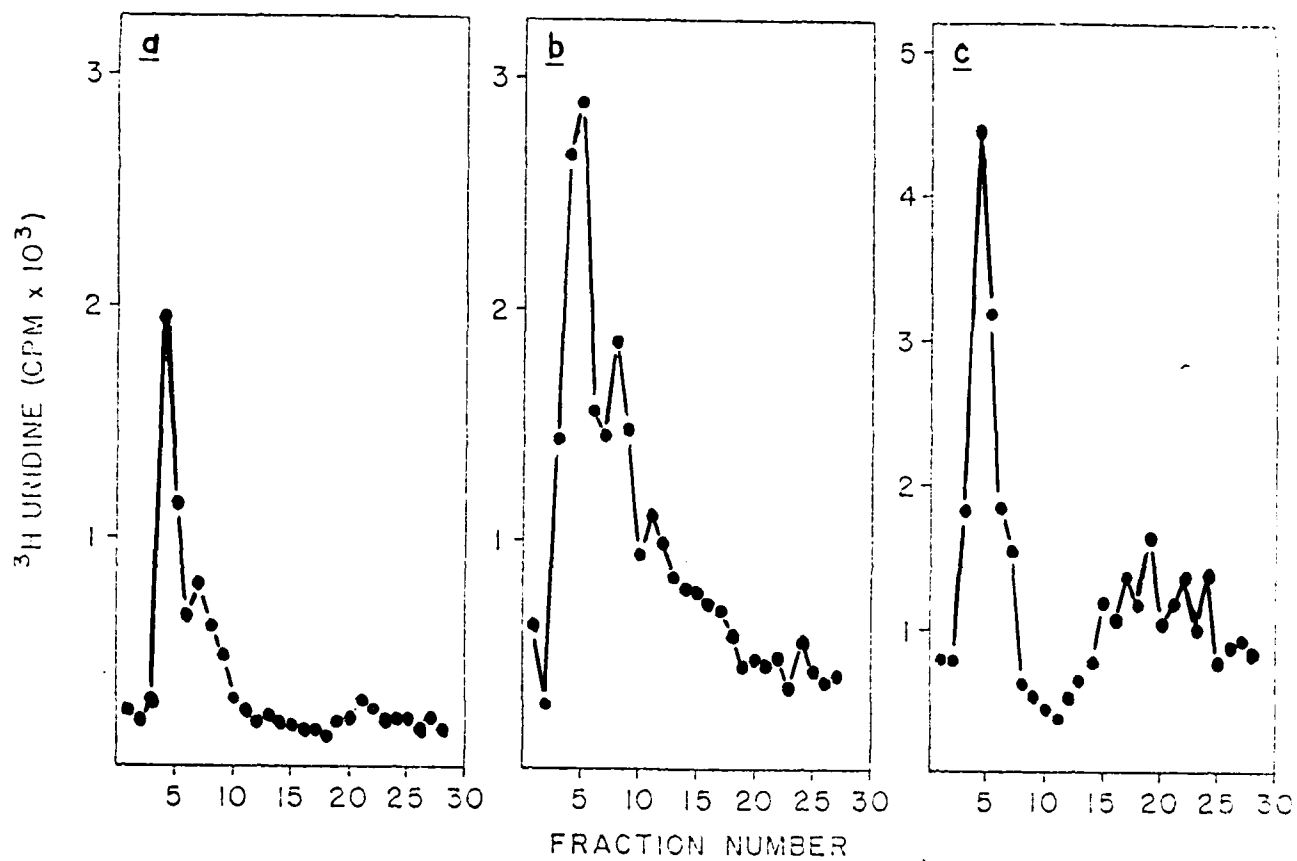
Figure 1

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Enclosure